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FINAL REPORT

TWO-GENERATION REPRODUCTION TOXICITY STUDY OF PROPYLTHIOURACIL WHEN ADMINISTERED TO SPRAGUE-DAWLEY RATS IN THE DRINKING WATER

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Study Completion Date:

To be added at report finalization

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Project Identification:

TRC Study No. 7244-601

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QUALITY ASSURANCE STATEMENT

(To be completed at the time of report finalization)

QUALITY ASSURANCE STATEMENT

COMPLIANCE STATEMENT

This study was conducted in compliance with the Good Laboratory Practice Regulations as set forth in Title 21 of the U.S. Code of Federal Regulations Part 58, issued December 22, 1978 (effective June 20, 1979). Standard Operating Procedure and protocol deviations occurred during the study and are documented in the study data; however, none of these deviations affected the quality or integrity of the study. To the best of my knowledge, this final report accurately describes the study methods and procedures used, and the reported results accurately reflect the raw data.

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_____

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ABSTRACT

Propylthiouracil was evaluated for potential reproductive toxicity using a Two-Generation study model. Beginning on Study Day (SD) 1, propylthiouracil was administered in the drinking water at dose levels of 0, 0.0001, 0.0004, and 0.0015 % (weight/volume) to adult male and female rats (N=20). The F_0 cohabitation period began on SD 71. Mating pairs were allowed to produce one litter. Dosing of the F_1 generation was initiated on post-natal day (PND) 21 (i.e., at weaning). On PND 99 ± 10, F_1 animals were assigned to mating pairs and allowed to produce one litter. Due to high mortality in the adolescent F_1 0.0015 % animals, this group was not available for the F_1 cohabitation. Endpoints evaluated included body weight, food and water consumption, clinical signs of toxicity, number and weight of pups, anogenital distance (AGD), sexual development endpoints, thyroid hormone levels, sperm parameters, vaginal cytology, organ weights, and gross and microscopic pathology.

In the F_0 Cohabitation there were significant decreases at the 0.0015% dose level in mean body weights (6-12%), mean food consumption (3-35%) expressed as g/kg body weight/day and g/animal/day, and water consumption (13-38%) expressed as g/animal/day. The pregnancy index was comparable in all groups, but the number of female and total pups was decreased by 43 and 30%, respectively, at 0.0015%. Pup weights were comparable among groups but by PND 14 there was a significant decrease (15-37%) in the 0.0015% male and female pup weights. Survival was also comparable until weaning on PND 21, after which an increase in mortality was observed at 0.0015%. By PND 25, the 0.0015% pups were smaller with domed heads and misshapen snouts. During necropsy on PND 21 a delay in eruption of teeth was noted. Microscopically the jaws had damage (depletion and vacuolation) to the odontoblasts and ameloblasts resulting in a delay in cellular maturation and subsequent tooth eruption.

In the F_1 offspring, eye opening was delayed by 1.9 days at 0.0015%. Prepuce separation was delayed by 2.0 days and vaginal opening by 1.4 days at 0.0004%. Because of the mortality observed in the F_1 animals at 0.0015%, only the 0, 0.0001, and 0.0004% groups continued to the F_1 cohabitation. In the F_1 cohabitation, no changes were noted in body weights, food consumption,

water consumption, or reproductive endpoints, except there was a decrease in anogenital distance noted in the males at 0.0001 and 0.0004%.

In the necropsy of F_0 parents there were significant decreases at 0.0015% in absolute adrenal, brain, kidney, liver, and spleen weights and relative right testis and seminal vesicle weights. The absolute thyroid/parathyroid weights were increased 67-373% in the 0.0004 and 0.0015% males and females; relative thyroid/parathyroid weights were increased 34-443% in the 0.0004 and 0.0015% males and females and 18% in the 0.0001% females. In the necropsy of the F_1 parents there were significant increases in the absolute and relative thyroid weights in the 0.0004% males and females and also in relative thyroid weight at 0.0001% (males only). The findings in the F_0 parents correlated with enlarged thyroids/parathyroids observed at necropsy in the 0.0004% males and females (7/20 and 1/20, respectively) and 0.0015% males and females (20/20 and 18/20, respectively). There were no gross pathology findings in the F_1 parents. Upon microscopic examination of the thyroid, follicular cell hyperplasia was observed in 10/10 0.0015% F_0 males and females, 7/10 0.0004% F_0 males, and 1/10 0.0004% F_1 males. There was also degeneration of the germinal epithelium of the testes in 2/10, 3/10, and 3/10 0.0001%, 0.0004%, and 0.0015% F_0 males respectively, and 1/10 0.0001% and 0.0004% F_1 males. TSH levels were increased in the 0.0004% and 0.0015% F_0 males and females and 0.0004% F_1 males and females. T4 levels were decreased in the 0.0004% and 0.0015% F_0 males and females and the 0.0004% F_1 males and females.

Based on the findings of this two generation study with one litter per generation, Propylthiouracil would be considered to be a reproductive/developmental toxicant in females at dose levels greater than or equal to 0.0004% based on decreased total pups per litter, delayed vaginal opening, delayed eye opening, and changes in estrous cyclicity. PTU would be considered a male reproductive toxicant at dose levels greater than or equal to 0.0001% based on delayed eye opening, delayed preputial separation, degeneration of the germinal epithelium of the testes, and decreased anogenital distance. Propylthiouracil would also be considered a general toxicant at 0.0015 and 0.0004% based upon decreased body weight and food consumption (mostly at 0.0015%) and increased thyroid weights, changes in thyroid hormone levels, and /or thyroid follicular cell hyperplasia at 0.0004 and 0.0015%.

SUMMARY OF RESULTS

TWO-GENERATION REPRODUCTION TOXICITY STUDY OF PROPYLTHIOURACIL WHEN ADMINISTERED TO SPRAGUE-DAWLEY RATS IN THE DRINKING WATER

Parameters	Treatment Group (%) Low (0.0001)	Mid (0.0004)	High (0.0015)
General Toxicity - F_0 Cohabitation			
Body Weight			
Male (Weeks 3 - 20)	-	-	↓
Female (Weeks 6, 7 and 9 - 20)	-	-	↓
Female (Week 16)	↓	-	↓
Sire at Delivery	-	-	↓
Dam at Delivery	-	↓	↓
Lactating Females (PND 4, 7 and 14)	-	-	↓
Feed Consumption (g/kg body weight/day)			
Male (Weeks 2 - 5, 8 and 10)	-	-	↓
Male (Week 13)	↓	↓	↓
Male (Weeks 6 - 7)	↓	-	↓
Male (Week 17)	↓	-	-
Female (Weeks 2 - 10)	-	-	↓
Lactating Females (PND 4 - 7)	↓	-	↓
Lactating Females (PND 11 - 14)	-	↑	-
Lactating Females (PND 18 - 21)	-	-	↓

KEY: M = Male
F = Female

↑ = Significant increase in the parameter.
↓ = Significant decrease in the parameter.
- = No observed effect.

SUMMARY OF RESULTS (CONTINUED)
TWO-GENERATION REPRODUCTION TOXICITY STUDY OF PROPYLTHIOURACIL WHEN ADMINISTERED TO SPRAGUE-DAWLEY RATS IN THE DRINKING WATER

Parameters	Treatment Group (%) Low (0.0001)	Mid (0.0004)	High (0.0015)
Feed Consumption (g/animal/day)			
Male (Weeks 2 - 10 and 13 - 19)	-	-	↓
Male (Week 13)	-	↓	↓
Female (Weeks 3 - 10 and 13 - 19)	-	-	↓
Female (Week 6)	-	↓	↓
Lactating Females (PND 1 - 4)	↓	-	↓
Lactating Females (PND 4 - 7)	↓	↓	↓
Lactating Females (PND 7 - 11 and 18 - 21)	-	-	↓
Lactating Females (PND 11 - 14)	-	↑	-
Water Consumption (g/kg body weight/day)			
Female (Week 18)	-	↓	↓
Lactating Females (PND 4 - 7, 11 - 14 and 18 - 21)	-	-	↓
Water Consumption (g/animal/day)			
Male (Weeks 3 - 10, 13 - 15 and 18 - 19)	-	-	↓
Male (Week 17)	↓	↓	↓
Female (Weeks 4, 8, 10, 13 and 19)	-	-	↓
Female (Week 18)	-	↓	↓
Lactating Females (PND 1 - 4, 4 - 7, 7 - 11 and 11 - 14)	-	-	↓
Lactating Females (PND 14 - 18 and 18 - 21)	↓	-	↓
Estrous Cyclicity			
Number of Females with Regular Cycle	-	-	↓
Relative Amount of Time Spent in Estrous Stages	-	-	*

KEY: M = Male
F = Female
↑ = Significant increase in the parameter.
↓ = Significant decrease in the parameter.
* = Significant change in the parameter
- = No observed effect.

SUMMARY OF RESULTS (continued)
TWO-GENERATION REPRODUCTION TOXICITY STUDY OF PROPYLTHIOURACIL WHEN ADMINISTERED TO SPRAGUE-DAWLEY RATS IN THE DRINKING WATER

Parameters	Treatment Group (%) Low (0.0001)	Mid (0.0004)	High (0.0015)
Male Necropsy			
Terminal Body Weight	-	-	↓
Absolute Thyroid/Parathyroid Weight	-	↑	↑
Relative Brain Weight	-	-	↑
Relative Pituitary Weight	-	↑	↑
Relative Right Testis Weight	-	-	↑
Relative Seminal Vesicle Weight	-	-	↑
Relative Thyroid/Parathyroid Weight	-	↑	↑
Female Necropsy			
Terminal Body Weight	-	-	↓
Absolute Adrenal Weight	-	-	↓
Absolute Brain Weight	-	-	↓
Absolute Kidney Weight	-	-	↓
Absolute Liver Weight	-	-	↓

KEY: M = Male
F = Female
↑ = Significant increase in the parameter.
↓ = Significant decrease in the parameter.
- = No observed effect.

SUMMARY OF RESULTS (CONTINUED)
TWO-GENERATION REPRODUCTION TOXICITY STUDY OF PROPYLTHIOURACIL WHEN ADMINISTERED TO SPRAGUE-DAWLEY RATS IN THE DRINKING WATER

Parameters	Treatment Group (%) Low (0.0001)	Mid (0.0004)	High (0.0015)
Female Necropsy			
Absolute Spleen Weight	-	-	↓
Absolute Thyroid/Parathyroid Weight	-	-	↑
Relative Thyroid/Parathyroid Weight	↑	↑	↑
Microscopic Findings			
Male Thyroid - Follicular Cell Hyperplasia	-	↑	↑
Male Testes - Germinal Epithelium Degeneration	↑	↑	↑
Female Thyroid - Follicular Cell Hyperplasia	-	↑	↑
Thyroid Hormones			
Male TSH	-	↑	↑
Male T4	-	↓	↓
Female TSH	-	↑	↑
Female T4	-	↓*	↓*
Sperm Data			
Morphology - % of Abnormal Sperm	↑	-	-
Mortality (Control) 0M,0F	1M,0F	0M,0F	0M,2F

KEY: M = Male
F = Female

↑ = Significant increase in the parameter.
↓ = Significant decrease in the parameter.
* = These decreases were not statistically significant at $p<0.05$.
- = No observed effect.

SUMMARY OF RESULTS (CONTINUED)

TWO-GENERATION REPRODUCTION TOXICITY STUDY OF PROPYLTHIOURACIL WHEN ADMINISTERED TO SPRAGUE-DAWLEY RATS IN THE DRINKING WATER

Parameters	Treatment Group (%) Low (0.0001)	Mid (0.0004)	High (0.0015)
Reproductive and Developmental Parameters - F_0 Cohabitation			
Live Pups per Litter	-	-	↓
Female	-	-	↓
Male and Female			
Average Pup Weights	-	-	↓
Male (PND 14 and 21)	-	-	↓
Female (PND 14 and 21)	-	↑	a
Day of Pup Eye Opening	-	↑	a
Day of Pup Vaginal Opening	-	↑	a
Day of Pup Preputial Separation			
F_1 Male PND 21 Necropsy	-	-	↓
Terminal Body Weight	-	-	↓
Absolute Brain Weight	-	-	↑
Relative Brain Weight	-	-	↓
Relative Spleen Weight	-	-	↓
Relative Thymus Weight			

KEY: M = Male
F = Female

↑ = Significant increase in the parameter.
↓ = Significant decrease in the parameter.
- = No observed effect.

a These parameters were not evaluated in the 0.0015% F_1 animals due to increased mortality.

SUMMARY OF RESULTS (CONTINUED)
TWO-GENERATION REPRODUCTION TOXICITY STUDY OF PROPYLTHIOURACIL WHEN ADMINISTERED TO SPRAGUE-DAWLEY RATS IN THE DRINKING WATER

Parameters	Treatment Group (%) Low (0.0001)	Mid (0.0004)	High (0.0015)[a]
F_1 Female PND 21 Necropsy			
Terminal Body Weight	-	-	↓
Absolute Brain Weight	-	-	↓
Relative Brain Weight	-	-	↑
Relative Spleen Weight	-	-	↓
Relative Thymus Weight	-	-	↓
General Toxicity - F_1 Cohabitation			
Feed Consumption (g/animal/day)			
Male (Week 3)	↓	-	
Lactating Female (PND 4 - 7 and 7 - 11)	↓	-	
Lactating Female (PND 18 - 21)	-	↓	
Water Consumption (g/animal/day)			
Lactating Female (PND 4 - 7)	↓	↓	
Lactating Female (PND 1 - 4, 7 - 11 and 11 - 14)	↓	-	
Male Necropsy			
Absolute Thyroid/Parathyroid Weight	-	↑	
Relative Thyroid/Parathyroid Weight	↑	↑	

KEY: M = Male
F = Female
↑ = Significant increase in the parameter.
↓ = Significant decrease in the parameter.
- = No observed effect.

a This dose level was discontinued for the F_1 Cohabitation due to high mortality observed among F_1 adolescents.

SUMMARY OF RESULTS (CONTINUED)
TWO-GENERATION REPRODUCTION TOXICITY STUDY OF PROPYLTHIOURACIL WHEN ADMINISTERED TO SPRAGUE-DAWLEY RATS IN THE DRINKING WATER

Parameters		Treatment Group (%) Low (0.0001)	Mid (0.0004)	High (0.0015)
Female Necropsy				
Absolute Thyroid/Parathyroid Weight		-	↑	
Relative Thyroid/Parathyroid Weight		-	↑	
Microscopic Findings				
Male Thyroid – Follicular Cell Hyperplasia		-	↑	
Male Testes – Germinal Epithelium Degeneration		↑	↑	
Thyroid Hormones				
Male TSH		-	↑	
Male T4		-	↓	
Female TSH		-	↑	
Female T4		-	↓	
Mortality				
Adolescent	(Control) 0M,0F	0M,0F	0M,0F	23M,20F
Adult	(Control) 1M,0F	0M,0F	0M,0F	
Reproductive and Developmental Parameters – F_1 Cohabitation				
Anogenital Distance (AGD)				
Male AGD		↓	↓	
AGD/Male Pup Weight Ratio		-	↓	

KEY: M = Male
F = Female
↑ = Significant increase in the parameter.
↓ = Significant decrease in the parameter.
- = No observed effect.

SUMMARY OF RESULTS (CONTINUED)
TWO-GENERATION REPRODUCTION TOXICITY STUDY OF PROPYLTHIOURACIL WHEN ADMINISTERED TO SPRAGUE-DAWLEY RATS IN THE DRINKING WATER

Parameters	Treatment Group (%) Low (0.0001)	Mid (0.0004)
F_1 Male PND 21 Necropsy		
Relative Spleen Weight	↑	-
F_1 Female PND 21 Necropsy		
Absolute Thymus Weight	-	↓
Relative Thymus Weight	-	↓

The F_1 cohabitation started on PND 99 ±10.

KEY: M = Male
F = Female
↑ = Significant increase in the parameter.
↓ = Significant decrease in the parameter.
- = No observed effect.

REPRODUCTIVE TOXICANT:
Male - Yes
Female - Yes

CLASSIFICATION:

		0.0001 %	0.0004 %	0.0015 %
Males	Reproductive Toxicity	+	+	+
	General Toxicity	-	+	+
Females	Reproductive/Developmental Toxicity	-	+	+
	General Toxicity	-	+	+

INTRODUCTION

Propylthiouracil (CAS No. 51-52-5) is a thyroid hormone-synthesis inhibitor and antithyroid agent for the treatment of hyperthyroidism. Propylthiouracil (PTU) has been shown to decrease T3 and T4 while increasing TSH. This study was designed to validate a Two-Generation Study Model proposed to identify potent and weak thyroid toxicants. The dose levels selected for the study were 0.0001, 0.0004, and 0.0015 % PTU (weight/volume). The high dose of 0.0015% was expected to result in increased TSH, decreased T3 and T4, thyroid pathology, and decreased growth. The remaining dose levels were expected to result in less toxicity with no effect at 0.0001% PTU.

METHODS

General Study Design

A schematic diagram of this study is presented in Figure 1. The F_0 Cohabitation consisted of a control group and three treated groups (20 pairs/group). F_0 males and females were administered propylthiouracil in the drinking water starting on SD 1 and continuing until necropsy. F_0 animals had body weights collected at randomization, weekly, and at littering and feed and water consumption measured weekly when housed individually. During the lactation period for the F_1 litter, F_0 females also had body weights measured on PND 1, 4, 7, 14, and 21 and feed and water consumption measured for PND 1-4, 4-7, 7-11, 11-14, 14-18 and 18-21. Physical examinations were performed at randomization, at initiation of dosing, and weekly thereafter. Prior to cohabitation, vaginal cytology was conducted for 14 days on the F_0 dams. Following ten weeks of premating exposure to propylthiouracil, the F_0 animals were housed as breeding pairs (1:1 ratio). Vaginal smears were examined daily for confirmation of mating. When sperm or plug positive or after 14 days of cohabitation, the females were separated from the males. The F_1 pups were reared by the dam until weaning on PND 21. On PND 1, 4, 7, 14, and 21 the pups were counted and weighed. All pups had their anogenital distance (AGD) and individual weight recorded on PND 1 and were examined for pinna detachment and eye opening beginning on PND 2. All male pups were examined for retained nipples on PND 12 and 13.

On PND 16, 1-2 males and 1-2 females from each litter were randomly selected for rearing for the F_1 mating trial. These animals were assigned a unique identification number which was tattooed on the tail. Observations of testicular descent (starting on PND 16), vaginal opening (starting on PND 25), and preputial separation (starting on PND 35) were conducted. On PND 21, animals selected for the F_1 cohabitation were separated from the dam and housed. Pups selected for the F_1 cohabitation period were administered propylthiouracil in the drinking water starting on PND 21 (dose levels of 0.0001, 0.0004, and 0.0015 % PTU). Body weights were collected weekly starting on PND 21.

Three additional males and three additional females from all F_1 litters (when available) were randomly selected on PND 16 for the PND 21 necropsy. On PND 21 these animals were necropsied, terminal body weights and organ weights were obtained, and tissues were saved for histopathologic evaluation. The remaining F_1 pups were euthanized and discarded without necropsy on PND 21.

After the lactation phase was completed, terminal body weights were obtained for all F_0 animals, then the animals were euthanized and necropsied, organ weights were obtained, sperm analyses were performed, and tissues were saved for possible histopathologic evaluation.

F_1 weanlings selected for the F_1 cohabitation were reared in same sex groups until PND 99 ±10 days of age, when twenty animals of each sex in each dose group were randomly assigned to breeding pairs (avoiding sibling matings) and cohabited. Vaginal smears were examined daily for confirmation of mating. When sperm or plug positive or after 14 days of cohabitation, the females were separated from the males. Once cohabited, F_1 animals had body weights collected weekly and at littering. Feed and water consumption were measured weekly when the animals were housed individually (i.e., not during cohabitation). During the lactation period for the F_2 litter, F_1 females also had body weights measured on PND 1, 4, 7, 14, and 21 and feed and water consumption measured for PND 1-4, 4-7, 7-11, 11-14, 14-18 and 18-21. Physical examinations were performed weekly.

The F_2 pups were reared by the dam until PND 21. On PND 1, 4, 7, 14, and 21, the pups were counted and weighed. All pups had their AGD and individual weight recorded on PND 1 and were examined for pinna detachment and eye opening beginning on PND 2. All male pups were

examined for retained nipples on PND 12 and 13. Vaginal cytology was conducted on the F_1 dams for 14 days beginning at least 4 days after the last F_2 PND 21.

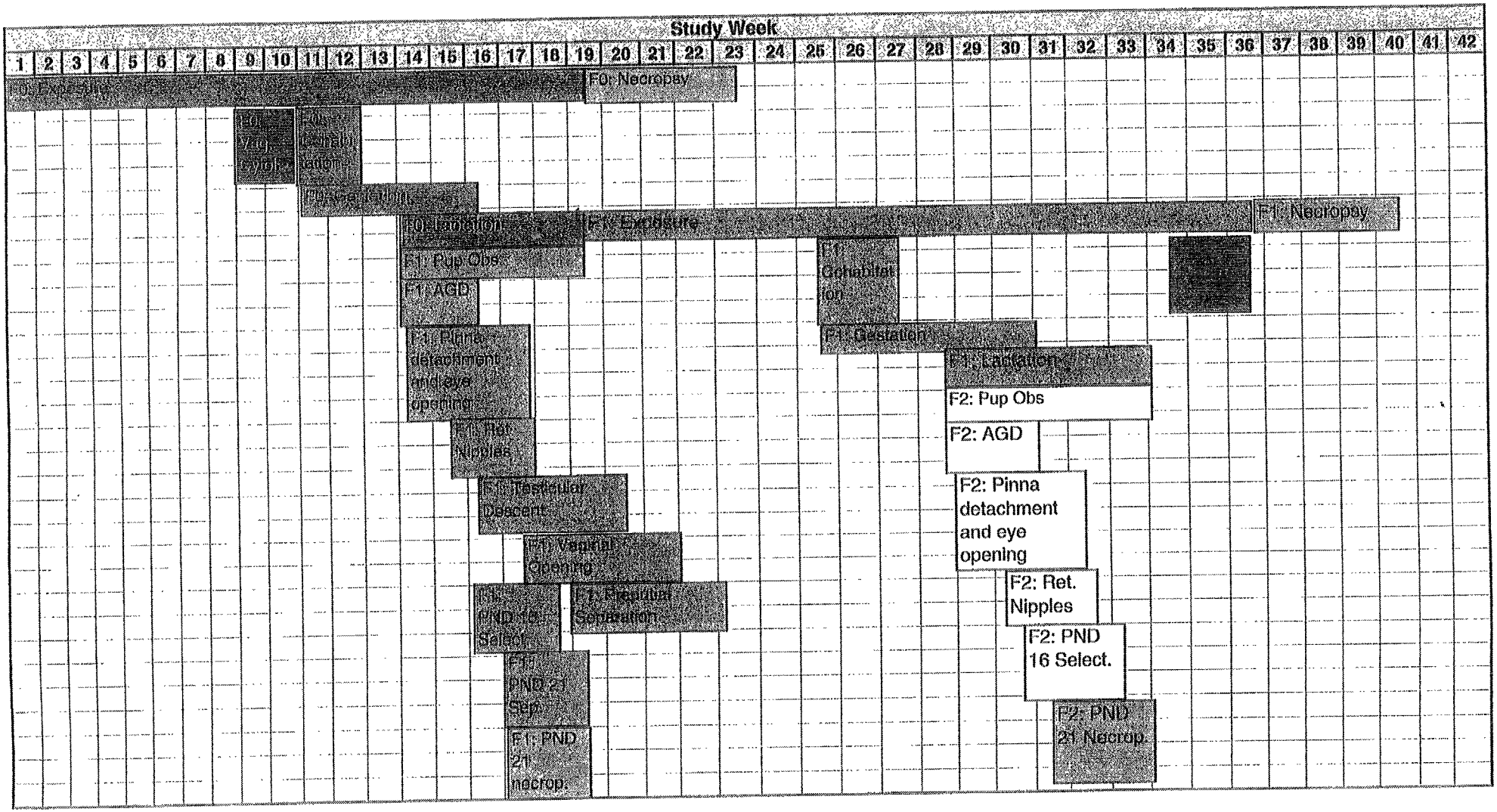
Three males and three females from all litters (where available) were randomly selected on PND 16 for the PND 21 necropsy. On PND 21 these animals were necropsied, terminal body weights and organ weights were obtained, and tissues were saved for histopathologic evaluation. The remaining F_2 pups were euthanized and discarded without necropsy on PND 21.

The F_1 animals were retained until completion of vaginal cytology. Terminal body weights were obtained for all F_1 animals, the animals were euthanized and necropsied, organ weights were obtained, sperm analyses were performed, and tissues were saved for possible histopathologic evaluation.

Figure 1

Two-Generation Reproduction Toxicity Study Flow Diagram for Propylthiouracil

Two-Generation Reproductio ity Flow Diagram for Propylthiouracil



Study Animals

The Sprague-Dawley rat was selected as the test animal due to its established quality as a breeder and the availability of historical toxicologic data for reference. For the F_0 cohabitation, 170 (85 male and 85 female) Sprague-Dawley Crl:CD® BR rats were received from Charles River Laboratories (Raleigh, NC) on May 9, 2000, and assigned temporary animal numbers. Seven days after receipt, all of the animals were weighed and randomly assigned to one of four groups by a computer-generated randomization procedure that ensured equal weight distribution between the groups. All study animals were assigned unique animal numbers and individually identified with the animal number on their tail by tattooing. Dosing was initiated after a 14 day acclimation period. During this period, two males and two females were forwarded to Anmed Biosafe, Inc., Rockville, Maryland, for determination of viral antibody titers. All sera were negative for antibodies at that time (Appendix table A2-4). Extra animals were subsequently removed from the study room. The study animals were approximately 7-8 weeks of age at the initiation of dosing and the body weight range of the animals was 232.3 – 293.9 g for males and 160.1 – 215.3 g for females.

The F_1 and F_2 animals were born at TherImmune Research Corporation. During the F_1 and F_2 litters, pup selection occurred on PND 16. On PND 16 for the F_1 litter, at least two males and two females from each litter, when available, were randomly selected for the F_1 cohabitation and identified with tail tattoos. On PND 21, these animals were housed separately from the dam, and dosing of the selected F_1 animals was initiated. On PND 16 for the F_1 and F_2 animals, three males and three females from each litter, when available, were randomly selected for the PND 21 necropsy. F_1 and F_2 pups not selected for PND 21 necropsy were euthanized by carbon dioxide asphyxiation and discarded without necropsy. For the F_1 animals, approximately one week before PND 99 ± 10 (initiation of F_1 cohabitation), one male was assigned to one female, avoiding sibling matings, to form twenty mating pairs per group for the F_1 cohabitation. Animals not selected for the F_1 cohabitation were euthanized via carbon dioxide asphyxiation and discarded without necropsy one week after the initiation of the F_1 cohabitation.

Animal Husbandry and Environmental Conditions

Animals were housed two per cage by sex from receipt to randomization, two per cage (one male and one female) during the F_0 and F_1 cohabitations, two per cage (same sex) during the F_1 growth phase, and individually at all other times. All animals were housed in polycarbonate cages (19"L x 10.5"W x 8"H) suspended on stainless-steel racks. Racks were equipped with filter paper liners. Polycarbonate caging contained Sani Chip certified heat treated hardwood laboratory bedding. Pelleted Harlan Teklad™ NIH-07 Small Animal Feed was available *ad libitum* in stainless-steel hanging feeders, and was used within five months of the milling date. The feed was analyzed for nutrients, aflatoxins, nitrosamines, heavy metals, chlorinated hydrocarbons, organophosphates, PCBs, nitrites, nitrates, BHA, BHT, total bacterial plates, coliforms, *E. coli*, and *Salmonella* by the vendor. Deionized water was provided in water bottles, which were changed at least weekly. The test article was given to the animals in the deionized water. The water is routinely analyzed for total dissolved solids, heavy metals, chlorinated hydrocarbons, organophosphates, nitrates, nitrites, microbiological content and total trihalomethanes at least semi-annually to conform with the Safe Drinking Water Act. None of the feed, water, or bedding contaminants was at levels believed sufficient to interfere with the study.

A 12-hour light/12-hour dark cycle was maintained throughout the study. During the study, the temperature range in the animal room was 68-74°F and the relative percent humidity was 30-70%, except as noted in Appendix Table A2-1.

Test Article

6-Propyl–2-thiouracil, lot no. 47H2500, was received from Battelle, Columbus, OH, on April 20, 2000 and June 16, 2000. It was described as a white powder with a purity of 99.8%. Prior to use, two 0.5 g samples of bulk test article were collected into glass bottles with Teflon® coated lids, sealed, and stored at approximately -20°C protected from light for possible future reanalysis. At the start of the F_1 cohabitation, and again after the end of the in-life portion of the study, five grams of bulk sample were sent to Battelle for analysis.

Each time a new mix or batch was prepared, two 50 mL archival samples of each dose level formulation were collected and stored at TherImmune in amber glass bottles with Teflon® coated

lids protected from light in the refrigerator. One sample of each dose level formulation from mixes 3, 4, 5, 11, 27, 51 was sent to Battelle for analysis. Archival samples which were not selected for analysis were discarded as hazardous waste at least 90 days after preparation.

Dosage Formulation and Administration

Formulations of 0, 0.0001, 0.0004, and 0.0015 % (w/v) were prepared at least weekly throughout the study. For Group 1 (control), a precalibrated carboy was filled with the required volume of deionized water. For all other groups, precalibrated carboys were initially filled to approximately 75% of the required volume with deionized water. The required quantity of 6-propyl-2-thiouracil was weighed into a weigh boat and poured into a volumetric flask (approximately half-filled with deionized water). The weigh boat was rinsed at least three times with deionized water and the rinse was added to the flask. The flasks were mixed until the propylthiouracil was completely dissolved. The solution was then poured into a carboy, the flask was rinsed at least three times with deionized water and the rinse was added to the carboy, deionized water was added to the carboy to achieve the required volume, and the solution was mixed with a variable speed stirrer to ensure complete dissolution.

The formulations were stored refrigerated and protected from light. Under these conditions, formulations are reported to be stable for 35 days. The formulations were dispensed into amber glass bottles with neoprene stoppers and stainless steel sipper tubes. Dispensed formulations are stable for seven days. The stability and storage of formulations conditions were based upon the Dose Formulation Developmental Study Report provided by Battelle.

F_0 Cohabitation

The F_0 cohabitation consisted of a control group and three treated groups (20 pairs/group). F_0 animals were administered propylthiouracil in their drinking water at doses of 0 (control), 0.0001, 0.0004, or 0.0015 % from SD 1 until necropsy. Prior to cohabitation, vaginal cytology was conducted for 14 days on the F_0 dams. Following ten weeks of premating exposure to propylthiouracil (i.e., on SD 71), the animals were housed as breeding pairs (1:1 ratio). Vaginal smears were examined daily for confirmation of mating. When sperm or plug positive or after 14

days of cohabitation, the females were separated from the males. The F_1 litter was reared by the dam until weaning on PND 21. The total number of pups, number of live and dead pups, the number of live male and female pups, and total body weight of live male and female pups were recorded on PND 1, 4, 7, 14, and 21. On PND 1, all pups had individual pup body weights and AGD measured, and the dam and sire weights were recorded. The dam was also weighed on PND 4, 7, 14 and 21, and feed and water consumption were measured for PND 1-4, 4-7, 7-11, 11-14, 14-18 and 18-21. All pups were examined for pinna detachment and eye opening beginning on PND 2. All male pups were examined for retained nipples on PND 12 and 13.

On PND 16, 1-2 males and 1-2 females from each litter were randomly selected for rearing for the F_1 mating trial. These animals were assigned a unique identification number and tail tattooed. Observations of testicular descent (starting on PND 16), vaginal opening (starting on PND 25), and preputial separation (starting on PND 35) were conducted. On PND 21, animals selected for the F_1 cohabitation were housed separately from the dam in same-sex pairs and drinking water dosing was initiated. Body weights were collected weekly starting on PND 21.

Three additional males and females from all litters (when available) were randomly selected on PND 16 for the PND 21 necropsy. On PND 21 these animals were necropsied, terminal body weights and organ weights were obtained, and tissues were saved for histopathologic evaluation. Pups not selected were euthanized by carbon dioxide asphyxiation and discarded without necropsy on PND 21.

Approximately one week before PND 99 ± 10 (i.e., the initiation of F_1 cohabitation), one male was assigned to one female, avoiding sibling matings, to form twenty mating pairs per group for the F_1 cohabitation. Animals not selected for the F_1 cohabitation were euthanized by carbon dioxide asphyxiation and discarded without necropsy.

All animals were observed twice daily for mortality and signs of toxicity. In males and non-lactating females, body weights were collected weekly and at littering (males only), and feed and water consumption was measured weekly when animals were housed individually. Physical examinations were performed weekly.

F_1 PND 21 Necropsy and Terminal Procedures

On PND 21, up to three surviving F_1 males and females per litter were weighed, sacrificed by carbon dioxide asphyxiation, and exsanguinated. A gross necropsy was performed on all animals. Necropsies were performed by trained personnel from Pathology Associates International (PAI, Frederick, MD) under the direct supervision of a Board-certified pathologist. Necropsies included examination of the external surface of the body, all orifices, and the cranial, thoracic, and abdominal cavities and their contents.

The following organs were weighed:

brain
spleen
thymus

The adrenals, kidneys, liver, pituitary, ventral prostate, dorsolateral prostate, seminal vesicles with coagulating glands, spleen, thyroid/parathyroids, vagina/cervix/uterus and gross lesions from all necropsied animals were preserved in 10% neutral-buffered formalin (NBF) for histopathological examination. The left testis and epididymis were fixed in a 2% para-formaldehyde/3% glutaraldehyde solution for 3-5 days and then transferred to phosphate buffered saline. The right testis was frozen at approximately -80°C to be used for spermatid head counts. (Spermatid head counts for PND 21 animals were discontinued because no sperm were found to be present in the testes of these animals.) The ovaries were preserved in Bouin's for 24-48 hours and then transferred to 70% ethanol. Histopathology was not required for these tissues.

F_0 Adult Necropsy and Terminal Procedures

After the end of lactation for all F_1 pups, terminal body weights were obtained from all surviving F_0 animals. Blood was collected from the orbital sinus under 70% O_2/30% CO_2 anesthesia. The blood was centrifuged and separated to obtain plasma. The plasma was frozen at approximately -80° and was forwarded to Anilytics, Inc. (Gaithersburg, MD) for determination of TSH, T3, and T4 levels. The methods used for the thyroid hormone analyses are presented in Appendix 7. Following blood collection, the animals were sacrificed by carbon dioxide asphyxiation and exsanguinated. A gross necropsy was performed on all animals. Necropsies were performed by trained personnel from PAI under the direct supervision of a Board-certified pathologist. Necropsies included examination of the external surface of the body, all orifices, and the cranial, thoracic, and abdominal cavities and their contents.

The following organs were weighed:

adrenals (paired)	brain
cauda epididymis (right)	epididymis (right)
kidneys (paired)	liver
ovaries (paired)	pituitary
prostate (ventral)	prostate (dorsolateral)
seminal vesicles with coagulating glands	spleen
testis (right)	thyroids/parathyroids
uterus/vagina/cervix	

The adrenals, brain, kidneys, liver, pituitary, ventral prostate, dorsolateral prostate, seminal vesicles with coagulating glands, spleen, thyroid/parathyroids, vagina/cervix/uterus, and gross lesions from all necropsied animals were preserved in 10% NBF for histopathological examination. The left testis and epididymis were fixed in a 2% para-formaldehyde/3% glutaraldehyde solution for 3-5 days and then transferred to phosphate buffered saline. The right testis was frozen at approximately -80°C and used for spermatid head counts. The ovaries were preserved in Bouin's for 24-48 hours and then transferred to 70% ethanol. The thyroid/parathyroids, ovaries, uterus, cervix, vagina, and gross lesions from the first ten surviving males and females per group were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically by a pathologist. The left testis and epididymis from the first ten surviving males per group were

processed through glycol methacrylate (GMA), sectioned, stained with periodic acid-Schiff (PAS) and hematoxylin, and microscopically evaluated by a pathologist.

Computer-assisted sperm motion analysis using Hamilton-Thorne Research Version 10 IVOS Sperm Analysis System (Hamilton-Thorne Research, Beverly, Massachusetts), epididymal sperm density, sperm morphology, and testicular spermatid head count data were measured on all males surviving to termination. The right vas deferens was used for sperm motility analysis; the right cauda epididymis was used for sperm density and morphology; and the right testis was used for evaluation of spermatid head counts. After conducting the epididymal sperm density counts, approximately 5mL of each sperm suspension was saved, frozen, and sent to an NTP subcontractor.

Animals found dead or euthanized *in extremis* during the study (all generations) were subject to a gross necropsy. The following tissues were retained in NBF for possible histopathological examination:

adrenals	brain
kidneys	liver
left epididymis	left testis
ovaries (transferred to 70% ethanol within 24-48 hours)	pituitary
	prostate (ventral and dorso-lateral lobes)
seminal vesicles/coagulating glands	spleen
stomach	thyroid/parathyroids
vagina/uterus/cervix	gross lesions

The lower and upper jaws of ten F_1 0.0015% (Group 4) animals (one animal/sex/litter) were embedded in paraffin, step sectioned, and examined microscopically by the study pathologist.

Allocation of F_1 Weanlings

On PND 16, 1 - 2 males and 1 - 2 females per litter, when available, were randomly selected for the F_1 cohabitation. These animals were assigned a unique identification number and tail tattooed. Observations of testicular descent (starting on PND 16), vaginal opening (starting on PND 25), and preputial separation (starting on PND 35) were conducted until the endpoint was seen. On PND 21, animals selected for the F_1 cohabitation were housed separately from the dam, and administration of propylthiouracil in drinking water was initiated. On PND 21, pups not selected for

the F_1 cohabitation or PND 21 necropsy were euthanized and discarded without necropsy. All animals were observed twice daily for mortality and signs of toxicity. Following weaning of the final F_1 litter, all weekly body weights and physical examinations were adjusted to occur on the same day and began on PND 29 ± 10.

Due to a high level of mortality in the 0.0015 % F_1 animals following weaning, all surviving animals in this group were removed from the study and euthanized.

F_1 Cohabitation

The F_1 cohabitation consisted of a control group and two treated groups (20 pairs/group). F_1 weanlings selected for the F_1 cohabitation were reared in same sex groups until PND 99 ± 10 days of age when twenty animals of each sex in each dose group were randomly assigned to breeding pairs (avoiding sibling matings) and cohabited. Vaginal smears were examined daily for confirmation of mating. When sperm or plug positive or after 14 days of cohabitation, the females were separated from the males. The F_2 litter was reared by the dam until PND 21. The total number of pups, number of live and dead pups, the number of live male and female pups, and total body weight of live male and female pups were recorded on PND 1, 4, 7, 14, and 21. On PND 1, all pups had individual pup body weights and AGD measured, and the dam and sire weights were recorded. The dam was also weighed on PND 4, 7, 14 and 21, and feed and water consumption were measured for PND 1-4, 4-7, 7-11, 11-14, 14-18 and 18-21. All pups were examined for pinna detachment and eye opening beginning on PND 2. All male pups were examined for retained nipples on PND 12 and 13. Weekly physical examinations and body weights were continued. In males and non-lactating females, body weights were collected weekly and at littering (males only), and feed and water consumption was measured weekly, when animals were housed individually. Physical examinations were performed weekly. All F_1 adults were observed twice daily for mortality and signs of toxicity. All animals not selected for the F_1 cohabitation were euthanized and discarded without necropsy on PND 106 ± 10.

Three males and three females from all litters (where available) were randomly selected on PND 16 for the PND 21 necropsy. On PND 21 for these animals, terminal body weights were

recorded, the animals euthanized and necropsied, organ weights were obtained, and tissues were saved for histopathologic evaluation.

Pups not selected were euthanized and discarded without necropsy on PND 21.

At least 4 days after the delivery of the last F_2 litters, vaginal cytology was conducted for 14 days on the F_1 dams.

F_2 PND 21 Necropsy and Terminal Procedures

Up to three surviving F_1 males and females per litter were weighed, sacrificed by carbon dioxide asphyxiation, and exsanguinated. A gross necropsy was performed on all animals. Necropsies were performed by trained personnel from PAI under the direct supervision of a Board-certified pathologist. Necropsies included examination of the external surface of the body, all orifices, and the cranial, thoracic, and abdominal cavities and their contents.

The following organs were weighed:

brain
spleen
thymus

The adrenals, kidneys, liver, pituitary, ventral prostate, dorsolateral prostate, seminal vesicles with coagulating glands, spleen, thyroid/parathyroids, vagina/cervix/uterus and gross lesions from all necropsied animals were preserved in 10% NBF for histopathological examination. The left testis and epididymis were fixed in a 2% para-formaldehyde/3% glutaraldehyde solution for 3-5 days and then transferred to phosphate buffered saline. The right testis was frozen at approximately -80°C to be used for spermatid head counts. (Spermatid head counts for PND 21 animals were discontinued because no sperm were found in the testes of these animals.) The ovaries were preserved in Bouin's for 24-48 hours and then transferred to 70% ethanol. Histopathology was not performed on these tissues.

F_1 Adult Necropsy and Terminal Procedures

After the conclusion of vaginal cytology for all F_1 females, terminal body weights were obtained from all surviving F_1 animals. Blood was collected from all animals from the orbital sinus under 70% O_2/30% CO_2 anesthesia. The blood was centrifuged and separated to obtain plasma. The plasma from each animal was then split into aliquots and frozen at approximately -80°. One aliquot was sent to Anilytics, Inc. (Gaithersburg, MD) for determination of TSH, T3, and T4 levels. The other aliquot was retained at TherImmune for possible future reanalysis. The methods used for the thyroid hormone analyses are presented in Appendix 7. Following blood collection, the animals were euthanized by carbon dioxide asphyxiation and exsanguinated. A gross necropsy was performed on all animals. Necropsies were performed by trained personnel from PAI under the direct supervision of a Board-certified pathologist. Necropsies included examination of the external surface of the body, all orifices, and the cranial, thoracic, and abdominal cavities and their contents.

The following organs were weighed:

adrenals (paired)	brain
cauda epididymis (right)	epididymis (right)
kidneys (paired)	liver
ovaries (paired)	pituitary
prostate (ventral)	prostate (dorsolateral)
seminal vesicles with coagulating glands	spleen
testis (right)	thyroids/parathyroids
uterus/vagina/cervix	

The adrenals, brain, kidneys, liver, pituitary, ventral prostate, dorsolateral prostate, seminal vesicles with coagulating glands, spleen, thyroid/parathyroids, vagina/cervix/uterus, and gross lesions from all necropsied animals were preserved in 10% neutral-buffered formalin for histopathological examination. The left testis and epididymis were fixed in a 2% para-formaldehyde/3% glutaraldehyde solution for 3-5 days and then transferred to phosphate buffered saline. The right testis was frozen at approximately -80°C and used for spermatid head counts. The ovaries were preserved in Bouin's for 24-48 hours and then transferred to 70% ethanol. The thyroid/parathyroids, ovaries, uterus, cervix, vagina, and gross lesions from the first ten surviving males and females per group were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically by a pathologist. The left testis and epididymis from the first

ten surviving males per group were processed through glycol methacrylate (GMA), sectioned, stained with periodic acid-Schiff (PAS) and hematoxylin, and microscopically evaluated.

Computer-assisted sperm motion analysis using Hamilton-Thorne Research Version 10 IVOS Sperm Analysis System (Hamilton-Thorne Research, Beverly, Massachusetts), epididymal sperm density, sperm morphology, and testicular spermatid head count data were measured on all males surviving to termination. The right vas deferens was used for sperm motility analysis; the right cauda epididymis was used for sperm density and morphology; and the right testis was used for evaluation of spermatid head counts. After conducting the epididymal sperm density counts, approximately 5 mL of each sperm suspension was saved, frozen, and sent to an NTP subcontractor.

Statistical Analyses

Data were statistically analyzed by Analytical Sciences Inc. (Durham, NC). Most hypotheses (including but not limited to pup weights, body weights, feed and water consumption, organ weights, sperm parameters, and estrous cycle length) were tested using the nonparametric multiple comparisons procedure of Dunn (1964) or Shirley (1977), as modified by Williams (1986). Shirley's test was designed to detect treatment-related differences when the response to treatment consistently increased (or decreased) with increasing dose. Although the test employs a smoothing algorithm to adjust for dose-response inversions, Dunn's test was more appropriate if the departure from monotonicity was severe. Jonckheere's test (1954) was used to ascertain whether there was sufficient evidence of a dose-related response to apply Shirley's test. If the p-value from Jonckheere's test was less than 0.01, Shirley's test was used; otherwise, Dunn's test was applied. Reproductive and developmental data were analyzed nested by dam.

For data expressed as a proportion, such as number fertile/number cohabitated, the Cochran-Armitage test (Armitage, 1971) was used to test for a dose-related trend, and pairwise comparisons were performed using a chi-squared test (Conover, 1971).

The ratio of the number of pups born alive to the total number of pups carried to full term was computed for all fertile pairs. The sex ratio, expressed as the proportion of males, was computed for all fertile pairs with at least one live pup. Shirley's or Dunn's test was used to compare dosed groups to controls based on Jonckheere's test, as described above.

Since the number of pups in a litter may influence the average pup weight, a parametric analysis of covariance (Neter and Wasserman, 1974) was used to test overall equality in average pup weight, after adjustment for average litter size. The covariant used was average litter size, including live and dead pups. Least squares estimates of dose group means adjusted for litter size were computed and tested for overall equality using an F-test and pairwise equality using Dunnett's test (1955). Unadjusted weights were analyzed with Shirley's or Dunn's test.

Absolute organ weights were analyzed using body weight as a covariate. Adjusted mean dose effects were compared to the control with Dunnett's test.

Litter sizes and number of litters in dose groups were compared to controls using Dunn's or Shirley's test. To examine potential differences in treatment effects on males and females, number of male pups, number of female pups, and total number of pups in litters in treated groups were compared to controls.

Using either Shirley's or Dunn's test, feed and water consumption data were analyzed as g/animal/day and g/kg body weight/day. Sperm parameters were analyzed by Shirley's or Dunn's test.

The relative time spent in estrous cycle stages was analyzed using a multivariate analysis of variance (Wilks criterion) to test for the simultaneous equality of measurements across dose levels (Morrison, 1976). Before applying the test, an arcsine transformation was performed to bring the data into closer conformance with normality assumptions.

Thyroid hormone data were analyzed using Dunnett's one-tailed test followed by Jonckheere's trend test.

All findings described in this report as "increased" or "decreased" were statistically significant as compared to the control group at the 95% confidence level ($p<0.05$).

Specimen, Raw Data and Final Report Storage

Upon submission of the final report, all original study records, including all original data sheets; the original final report; all biological samples; tissues; sperm morphology slides; sperm data optical disks; computer printouts generated in the statistical analysis of the sperm data; and copies of the final report, will be forwarded to the contracting agency, the NIEHS, Research Triangle Park, NC. Copies of the final study report will also be filed with TherImmune Research Corporation.

RESULTS

F_0 Cohabitation

During Weeks 3-20 of the F_0 Cohabitation, mean body weights of the 0.0015% males were decreased by 6-21% (Table 0-1A). Mean body weights of the 0.0015% females were decreased by 8-17% during Weeks 6-7 and 9-20. Mean body weights for the 0.0001% females were decreased by 6% during Week 16 (Table 0-1B).

Feed consumption was decreased by 4-11% during Weeks 6-7, 13, and 17 in the 0.0001% males, by 11% during Week 13 in the 0.0004% males, by 6-18% during Weeks 2-8, 10, and 13 in the 0.0015% males, and by 3-17% during Weeks 2-10 in the 0.0015 % females, when examined as g/kg body weight/day (Table 0-2A). Feed consumption examined as g/animal/day was decreased by 12% during Week 13 in the 0.0004% males, by 12-35% during Weeks 2-10 and 13-19 in the 0.0015% males, by 9% during Week 6 in the 0.0004% females, and by 12-25% during Weeks 3-10, 13, and 18-19 in the 0.0015% females (Table 0-2B).

Water consumption was decreased in the 0.0004 and 0.0015% females by 15 % and 31%, respectively, during Week 18 when examined as g/kg body weight/day (Table 0-3A). When examined as g/animal/day, water consumption was decreased by 16% during Week 17 in the 0.0001 and 0.0004% males, by 14-23% during Weeks 2-10, 13-15, and 17-19 in the 0.0015% males, by 18% during Week 18 in the 0.0004% females, and by 13-38% during Weeks 4, 8, 10, 13, and 18-19 in the 0.0015% females (Table 0-3B).

Clinical signs noted in the F_0 animals included abrasions, alopecia, anorexia, discharge from the eye, hunched posture, lacrimation, languid behavior, loss of appetite, rough haircoat, swelling, vaginal discharge, and thinness. The incidence of these observations was low (0-10%), with the exception of alopecia (10-45%), and was not considered to be treatment-related (Appendix Tables A0-4A and A0-4B).

The estimated dosage for the parental generation was 0.1 mg/kg/day for Group 2, 0.2 to 0.5 mg/kg/day for Group 3, and 0.9 to 1.8 mg/kg/day for Group 4 (Table 0-4). The estimated dosage levels for the F_1 offspring were 0.1 to 0.3 mg/kg/day for Group 2, 0.5 to 1.3 mg/kg/day for Group 3, and 1.9 to 3.4 mg/kg/day for Group 4 (Table 0-9).

The following changes were observed in the reproductive data for the F_0 cohabitation:

- Live pups per litter were decreased by 43 and 30% for female and total pups, respectively, in the 0.0015% group (Table 0-5).
- Average pup weights at PND 14 and 21 were decreased by 15-33% for males and 15-37% for females in the 0.0015% group (Table 0-11).
- Sire weights at delivery for the 0.0015% males were decreased by 20% (Table 0-5).
- Body weights for lactating 0.0015% dams were decreased by 14-18% on PND 1, 4, 7, and 14. Body weights for lactating 0.0004% dams were decreased by 7% on PND 1 (Table 0-5 and Table 0-6).
- Feed consumption in lactating dams was decreased by 26% on PND 4-7 for 0.0001% dams and by 32-42% on PND 4-7 and 18-21 for 0.0015% dams and was increased by 25% on PND 11-14 for 0.0004% dams when examined as g/kg body weight/day (Table 0-7A).
- Feed consumption in lactating dams was decreased by 32-36% on PND 1-4 and 4-7 for 0.0001% dams, by 16% on PND 4-7 for 0.0004% dams, and by 31-49% on PND 1-4, 4-7, 7-11, and 18-21 for 0.0015% dams and was increased by 22% on PND 11-14 for 0.0004% dams when examined as g/animal/day (Table 0-7B).
- Water consumption in lactating dams was decreased by 28-40% on PND 4-7, 11-14, and 18-21 for 0.0015% dams when examined as g/kg body weight/day (Table 0-8A).
- Water consumption in lactating dams was decreased by 11-16% on PND 14-18 and 18-21 for 0.0001 % dams and by 25-39% throughout lactation for 0.0015% dams when examined as g/animal/day (Table 0-8B).
- The day of eye opening, day of vaginal opening and day of preputial separation were all delayed in the 0.0004% group (1.9 days, 1.4 days and 2.0 days, respectively) (Tables 0-14 and 0-15).
- There was no change in anogenital distance in the F_1 males or females.

Vaginal cytology was performed on F_0 dams for 14 days prior to cohabitation. The percentage of females with regular cycles was decreased from 95% in the controls to 70% in the 0.0015 % females, and these animals also differed from the controls in the relative amount of time spent in estrous stages. No changes were seen in cycle length, number of cycles, or number of cycling females across the dose groups as compared to the control females (Table 0-20).

Mortality was observed in one F_0 males and two F_0 females. One 0.0001 % male was sacrificed as moribund on SD 113 after observations of anorexia, thinness, rough haircoat, languid behavior, and hunched posture. Two 0.0015 % females were found dead on SD 98 and 110. These females appeared normal prior to death (Table 0-27).

F_1 PND 21 Necropsy

In the 0.0015% males, terminal body weights decreased by 23%. Absolute brain weights were decreased by 6%, and relative spleen and thymus weights were decreased by 15 and 41%, respectively. Relative brain weights were increased by 16% (Table 0-16). All absolute and relative organ weights in the 0.0001 and 0.0004% males were comparable to controls. Incidental gross findings at necropsy consisted of dilated kidneys, a discoloration on a mammary gland, and masses and a nodule on the thymus (Table 0-17).

In the 0.0015% females, terminal body weights decreased by 26%. Absolute brain weights were decreased by 7%, and relative spleen and thymus weights were decreased by 17 and 42%, respectively. Relative brain weights were increased by 17% (Table 0-18). All weights in the 0.0001 and 0.0004% females were comparable to controls. Incidental gross findings at necropsy consisted of dilated kidneys and a small spleen (Table 0-19).

Histopathologic examination was not performed on these tissues.

F_0 Thyroid Hormone Analysis

TSH levels in the 0.0004% and 0.0015% males increased by 126% and 510%, respectively. T4 levels decreased by 46% in the 0.0004% males and by 78% in the 0.0015% males. TSH levels in the 0.0004% and 0.0015 % females increased by 91% and 476%, respectively. T4 levels decreased

by 52% in the 0.0004% females and by 49% in the 0.0015% females (non-significant). T3 levels for all treated groups were comparable to controls (Table 0-21).

F_0 Adult Necropsy

In the F_0 0.0004 and 0.0015% males, the absolute thyroid/parathyroid weight was increased by 67% and 301%, respectively. In the 0.0015% males, terminal body weight was decreased by 19%, while relative brain (15%), right testis (23%) and seminal vesicle (16%) weights were all increased. In the F_0 females, the absolute thyroid/parathyroid weight was increased in the 0.0015% group by 373%, while relative thyroid/parathyroid weights were increased in the 0.0001, 0.0004, and 0.0015% animals (18, 34, and 443% respectively). In the 0.0015% females, terminal body weights (13%) and absolute adrenal (22%), brain (6%), kidney (12%), liver (10%), and spleen (15%) weights were all decreased. All other weights (absolute and relative) for both males and females were comparable to the controls (Tables 0-23 and 0-26).

The percentage of abnormal sperm increased from 0.3% in the controls to 1.0% in the 0.0001% males. The sperm per mg of cauda, total sperm per cauda, spermatids per mg of testis, and total spermatids per testis for all treated groups were comparable to controls (Table 0-24A). Computer-assisted sperm motion analysis using Hamilton Thorne Integrated Visual Optic System revealed no changes in mean path velocity, progressive velocity, track speed, lateral amplitude, beat frequency, straightness, linearity, or motile percentage of the treated groups as compared to the controls (Table 0-24B).

Enlarged thyroids/parathyroids were seen in 0.0004% males (7 of 20), 0.0015% males (20 of 20), 0.0004% females (1 of 20), and 0.0015% females (18 of 20). Incidental gross findings in the males at necropsy included a discoloration on adipose tissue, a kidney cyst, and a discoloration on a mammary gland. Incidental findings in the females at necropsy included an enlarged kidney, a discoloration on a mammary gland, enlarged mammary glands, an enlarged pituitary, an enlarged urinary bladder, and distended, enlarged or fluid-filled vagina/cervix/uterus. All findings except the enlarged thyroids/parathyroids were spread throughout the dose groups and the incidence and severity were not related to dose (Tables 0-22 and 0-25).

Microscopic findings are described in the Pathology Report (Appendix 6). Thyroid follicular cell hyperplasia was seen in 0.0015% males (10 of 10) and females (10 of 10) and in 0.0004% males (7 of 10) and females (1 of 10). In addition, a follicular cell adenoma was seen in one of the 0.0015% females with follicular cell hyperplasia. Degeneration of the germinal epithelium of the testes was seen in 0.0015% males (2 of 10), 0.0004% males (3 of 10), and 0.0001% males (3 of 10). Multinucleated giant cells were only seen in 1 of the 0.0015% males exhibiting degeneration of the germinal epithelium.

F_1 Growing Phase

Increased mortality among the F_1 offspring was observed during the growing phase (i.e., after weaning): 23/29 males and 20/26 females in the 0.0015% group were either found dead or sacrificed as moribund.

By PND 25, the 0.0015% pups appeared to be smaller than pups in other groups, with domed heads and misshapen snouts. Six male and eight female pups had been found dead and 17 males and 12 females had been sacrificed as moribund (Table 1A-2). During necropsy of these unscheduled deaths, a delay in eruption of teeth was noted. The upper and lower jaws were evaluated microscopically. Changes were seen which seemed to represent damage (depletion and vacuolation) to the odontoblasts and ameloblasts, resulting in a delay in cellular maturation and subsequent tooth eruption. Tissues as examined, although not normal, seemed to be progressing toward normal, in spite of the delayed tooth eruption.

Because of the high level of mortality, the remainder of the 0.0015% pups were removed from the study and euthanized, and the growing phase and F_1 cohabitation were conducted with only three groups: control, 0.0001%, and 0.0004%.

There were no changes in male or female body weights during the F_1 growing phase (Table 1A-1).

Clinical signs noted during the growing phase included abrasions and alopecia. The incidence of these observations was low (0-17%), and was not considered to be treatment-related (Appendix Tables A1-2A and A1-2B). With the exception of the high-dose animals discussed above, there was no mortality seen during the growing phase (Table 1A-2).

F_1 Cohabitation

Mean body weights for all treated males and females were comparable to controls (Tables 1-1A and 1-1B).

All feed consumption values were comparable to controls when examined as g/kg body weight/day (Table 1-2A). When examined as g/animal/day, the feed consumption for 0.0001 % males decreased 8% during Week 4 (Table 1-2B). With the exception of lactating females, all water consumption values were comparable to controls when examined as either g/kg body weight/day or g/animal/day (Tables 1-3A or 1-3B).

Clinical signs noted during this portion of the study included abrasions, alopecia, swelling, a small stationary tissue mass, and an ulceration. The incidence of these observations was low to moderate (0-30%), and was not considered to be treatment-related (Appendix Tables A1-7A and A1-7B).

The following changes were observed in the reproductive data for the F_1 Cohabitation:

- Male anogenital distance (AGD) in the 0.0001 and 0.0004% pups was decreased by 8% and 7% respectively. Male AGD/Pup Weight Ratio in the 0.0004% pups was decreased by 8% (Table 1-10).
- Feed consumption in lactating dams was decreased by 11-12% on PND 4-11 for 0.0001% dams and by 9% on PND 18-21 for 0.0004% dams when examined as g/animal/day (Table 1-7B).
- Water consumption in lactating dams was decreased by 10-16% on PND 1-14 for 0.0001% dams and by 12% on PND 4-7 for 0.0004% dams when examined as g/animal/day (Table 1-8B).

Vaginal cytology was performed on F_1 dams for 14 days after the last F_2 litters. No changes were revealed in the number of females with regular cycles, cycle length, number of cycles, or number of cycling females across the dose groups as compared to the control females. Treated females did not differ from the control females in the relative amount of time spent in estrous stages (Table 1-19).

Mortality was observed in one F_1 animal. One control male was found dead on SD 67 of the F_1 cohabitation. The animal appeared normal prior to death (Table 1-26).

F_2 PND 21 Necropsy

In the 0.0001% males, the absolute spleen weight was increased by 14%, and the relative spleen weight was increased by 12%. All weights in the 0.0004% males were comparable to controls (Table 1-16). The only gross finding at necropsy was an enlarged liver in one control male (Table 1-15).

In the 0.0004% females, absolute and relative thymus weights were decreased by 9%. All weights in the 0.0001% females were comparable to controls (Table 1-18). There were no gross findings at necropsy (Table 1-17).

Histopathologic examination was not performed on these tissues.

F_1 Thyroid Hormone Analysis

TSH levels in the 0.0004% males increased by 172%. T4 levels in the 0.0004% males decreased by 61%. TSH levels in the 0.0004% females increased by 144%. T4 levels in the 0.0004% females decreased by 59%. T3 levels for all treated groups were comparable to controls (Table 1-20).

F_1 Adult Necropsy

Absolute thyroid/parathyroid weights increased by 80% in the 0.0004% males. Relative thyroid/parathyroid weights in the 0.0001 and 0.0004% males increased by 26 and 84%, respectively. Absolute and relative thyroid/parathyroid weights increased by 72 and 74%, respectively, in the 0.0004% females. All other weights (absolute and relative) for both males and females were comparable to the controls (Tables 1-22 and 1-25).

Computer-assisted sperm motion analysis using Hamilton Thorne Integrated Visual Optic System revealed no changes in mean path velocity, progressive velocity, track speed, lateral amplitude, beat frequency, straightness, linearity, or motile percentage of the treated groups as compared to the control (Table 1-23B). The total sperm per cauda, sperm per mg of cauda,

spermatids per mg of testis, total spermatids per testis, and percent of abnormal sperm were also all comparable to controls (Table 1-23A).

The only gross finding seen in a male at necropsy was a distended, red urinary bladder in one 0.0001% animal (Table 1-21). There were no gross findings in the females at necropsy (Table 1-24).

Microscopic findings are described in the Pathology Report (Appendix 6). Thyroid follicular cell hyperplasia was seen in one 0.0004 % male. Degeneration of the germinal epithelium of the testes was seen in one 0.0001 % male and one 0.0004 % male. There were no treatment-related microscopic changes in any of the F_1 females evaluated (Appendix 6).

DISCUSSION

In this two-generation study, propylthiouracil (PTU) was administered to male and female Sprague-Dawley rats in the drinking water at dose levels of 0.0001, 0.0004, or 0.0015 % (w/v). The corresponding dose levels were 0.1, 0.2 to 0.5, and 0.9 to 1.8 mg/kg/day, respectively. The F_0 generation was exposed to PTU only through their drinking water; the F_1 generation was exposed during gestation and lactation and subsequently through the drinking water; and the F_2 pups were exposed during gestation and lactation. Each generation was evaluated for general and reproductive/developmental toxicity endpoints.

In the F_0 males and females, body weights were decreased at 0.0015% but were comparable to controls at 0.0001 and 0.0004%. This decrease in body weight was previously observed by O'Connor *et al.* (1999) in males following 15 doses of PTU at 10 mg/kg. A similar effect was not seen in females following five doses. The body weight loss noted in the 0.0015% males and females was accompanied by decreased food and water consumption. Comparable decreases in body weight and food and water consumption were seen in lactating F_0 dams at 0.0015% and, to a much lesser extent, at 0.0001%. In general, less remarkable changes in body weight, food consumption, and water consumption were noted in the 0.0004% and, sporadically, the 0.0001% groups at some time points, indicating mild to moderate general toxicity at these doses.

Changes in estrous cyclicity were observed in the F_0 females at 0.0015% but not at the lower dose levels. These changes consisted of a decrease in the number of females with a regular cycle and changes in the amount of time spent in the various estrous stages. These changes did not affect female fertility, as no decrease in pregnancy index was noted among these animals.

Even though fertility was unaffected in the F_0 parents, there was a decrease in the number of female and total F_1 pups at 0.0015%. F_1 pup survival at this dose level was comparable through PND 21, although a marked decrease in male and female pup body weights was observed on PND 14 and became severe by PND 21. A high level of mortality was observed in these animals after weaning. By PND 25 the 0.0015% pups were noticeably smaller with domed heads and misshapen snouts. During the PND 21 necropsy a delay in eruption of the teeth was noted and the jaws, when evaluated microscopically, revealed depletion and vacuolization of the odontoblasts and ameloblasts, resulting in a delay in cellular maturation and subsequent tooth eruption. Tissues examined at PND

21, although not normal, seemed to be progressing toward normal, in spite of the delayed tooth eruption. Also noted at PND 21 were decreases in relative spleen, thymus, and brain weights and absolute brain weights in the 0.0015% males and females. These data reflect the overall diminished growth that was observed in these animals.

Due to the increased mortality observed among the F_1 juveniles at 0.0015%, surviving animals in this group were removed from study, and the remainder of the study was conducted using only 0, 0.0001, and 0.0004% dose levels.

In the F_1 offspring there were delays in eye opening (0.0015 %), preputial separation (0.0004 %), and vaginal opening (0.0004 %). No animals could be evaluated at 0.0015% for preputial separation and vaginal opening due to mortality. Preputial separation was delayed 2.0 days from 42.7 days in controls to 44.7 days at 0.0004%, and vaginal opening was delayed 1.4 days from 32.4 days in controls to 33.8 days at 0.0004 %. Marty *et al.* (2001) confirmed a delay in preputial separation in males dosed at 240 mg/kg/day from 44.4 days in controls to greater than 50 days in treated animals. Marty *et al.* (1999) confirmed that vaginal opening was delayed in females dosed at 240/mg/kg/day to 34.2 days from 32.3 or 33.5 days in controls. Wilen *et al.* (1981) also noted a delay in vaginal opening at 0.1% in immature rats fed PTU from weaning through the day of vaginal opening.

Delayed vaginal opening indicates delayed pubertal onset in juvenile female rats (Marty, 1999) and may occur with compounds that cause endocrine disruption or other reproductive toxicity. However, decreases in body weight may also delay vaginal opening, thereby making it more difficult to attribute delayed vaginal opening to reproductive/developmental toxicity or endocrine disruption (Marty, 1999). For compounds that have not been as thoroughly evaluated as PTU, for example, it is important to evaluate the significance of delayed vaginal opening in context with other observations of toxicity.

The observed delays in preputial separation and vaginal opening did not affect the subsequent fertility of the F_1 animals; no decreases in pregnancy index were noted among these animals during the F_1 Cohabitation. In contrast to the F_0 Cohabitation, no decreases in number of pups or pup weights were observed in the F_1 Cohabitation. However, decreased anogenital distance was noted in the 0.0004% and 0.0001% F_2 male pups, which was not observed in the F_1 male pups. The

AGD/pup weight ratio was also decreased in the 0.0004% pups. These observations are a definitive indicator of male reproductive toxicity at 0.0004% and possibly 0.0001%. Moreover, the decrease in AGD that was only seen in the F_2 pups supports a two-generation study design.

As expected, TSH levels were increased and T4 levels were decreased in the PTU-treated F_0 and F_1 animals. The TSH and T4 levels were correlated with the thyroid weights, discussed below, by being most affected at 0.0015%, less in the females, and more in the F_1 males and females. T3 levels were not affected by PTU administration. Analysis of T3 levels may be useful when evaluating other compounds, although it was not essential here.

In the PTU-treated F_0 and F_1 animals there were dose-related increases in absolute and relative thyroid weights. At 0.0015% the thyroid weight increases were 3 to 4 times the control weight, while at 0.0004% the increases were much less remarkable. The observed changes in thyroid weight were less severe in females than males and more severe in both sexes in the F_1 than the F_0 adults. At 0.0001% there was an increase in relative thyroid weight in the F_1 males and F_0 females. O'Connor *et al.* (1999) concluded that relative thyroid weight was a more sensitive indicator of thyroid toxicity than absolute thyroid weight: Relative thyroid weight removes the potentially confounding effect of body weight and allows detection of a true compound effect, particularly when decreases in body weight are observed. Also of interest is the observation of increased relative pituitary weights in the 0.0015% and 0.0004% F_0 males. The significance of this particular observation is unclear at this time, but may reflect increased demand on the pituitary for TRH.

At the F_0 gross necropsy, enlarged thyroids/parathyroids were seen in most 0.0015% animals and in 7/20 and 1/20 0.0004% males and females, respectively. No changes were noted in the F_1 gross necropsy findings.

Histopathology findings in the thyroid and testes were generally consistent between the F_0 and F_1 animals. Thyroid follicular cell hyperplasia was seen in all 0.0015% F_0 animals and in 7/10 0.0004% F_0 males, but in no F_1 females. In the 0.0004% animals, only 1/10 F_1 males was noted with thyroid follicular cell hyperplasia. The paucity of microscopic findings in the F_1 animals was surprising in light of the changes seen in thyroid weight and thyroid hormones and the microscopic findings in the F_0 animals. The pathology, thyroid hormone, and thyroid weight findings are in general agreement with those of O'Connor *et al.* (1999), Marty *et al.* (1999), and Marty *et al.* (2001).

O'Connor *et al.* (1999) observed these effects in females at 10 mg/kg/day when treated for five days and in males at 0.25 mg/kg/day when treated for 15 days. Marty *et al.* (1999) and Marty *et al.* (2001) treated females for 20 days at 240 mg/kg/day and males for 30 days at 240 mg/kg/day and observed similar findings. However, the belief that histopathology of the thyroid is the most sensitive indicator of thyroid toxicity (O'Connor, 1999) is not supported by the data presented here.

Degeneration of the germinal epithelium of the testes was also noted in all available treated male groups in the F_0 and F_1 generations. Degeneration was noted in 2/10, 3/10, and 3/10 F_0 males at 0.0015, 0.0004 and 0.0001%, respectively. In the F_1 males, degeneration was noted in 1/10 males at 0.0004 and 0.0001%.

Stoker *et al.* (2000) concluded that the induction of hypothyroidism by administration of PTU in polychlorinated biphenyls from birth to weaning increased testis size, number of Sertoli cells and Leydig cells, and daily sperm production in the adult. Other reports cited by Stoker *et al.* (2000) show that the critical period for this effect is the first two weeks after birth with no effects observed if PTU treatment was started during late lactation. Whether these results are attributable to the PTU or the PCBs is unknown. Regardless, the results in our study are in general agreement with these authors: There was no difference noted in the sperm parameters in either the F_0 males of the F_1 males.

Based on the findings of this two generation study with one litter per generation, propylthiouracil is considered to be a reproductive/developmental toxicant in females at dose levels greater than or equal to 0.0004% based on decreased total pups per litter, delayed vaginal opening, delayed eye opening, and changes in estrous cyclicity. PTU is considered a male reproductive toxicant at dose levels greater than or equal to 0.0001% based on delayed eye opening, delayed preputial separation, degeneration of the germinal epithelium of the testes, and decreased anogenital distance. Propylthiouracil is also considered a general toxicant at 0.0015 and 0.0004% based upon decreased body weight and food consumption (mostly at 0.0015%) and increased thyroid weights, changes in thyroid hormone levels, and/or thyroid follicular cell hyperplasia at 0.0004 and 0.0015%.

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